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TITLE: **Studies of the Tumor Microenvironment in Pathogenesis of Neuroblastoma**

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14. ABSTRACT The NBL-Tag neuroblastoma tumors were assessed for presence of macrophages and their role in promoting tumor growth. Anti-IL6 antibody therapy on a co-culture of macrophages and NBL-Tag tumor cell line (NBT2) did not decrease the tumor-promoting effects of 'trained' macrophages. Treatment of mice with macrophage depleting agents did not effect the total number of macrophages within the tumor-microenvironment, suggesting alternative strategies such as reversing the polarization of macrophages may be necessary. In vitro and in vivo experiments utilizing macrophages trained in vitro (via co-culture with tumor cells) or trained in vivo (obtained from					
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Introduction:

Neuroblastoma is the most common extra cranial solid tumor of childhood, and 45% of patients have high-risk tumors, nearly all of which are metastatic (stage 4) when diagnosed. Seventy-one percent of metastatic (stage 4) neuroblastomas (mNBL) lack amplification of the MYCN oncogene. An intriguing observation about survival of patients with MYCN non-amplified mNBL (mNBL MNA) is the extreme variation based on age at diagnosis. Children diagnosed less than 18 months of age have greater than 90% overall survival (O.S.), while those diagnosed after 18 months of age have only 45% O.S. even with improvements in therapy during the past 20 years. Recently, our group has identified several inflammation-related genes correlating with age at diagnosis and outcome in this group of tumors. Our characterization of a recently described 100% penetrant transgenic murine neuroblastoma model (NBL-Tag) established lack of MYCN amplification using comparative genomic hybridization (aCGH). Remarkably, tumors are not detected by MRI until 13 weeks of life, but they then grow rapidly with liver and bone marrow metastasis by 20 weeks and demise by 28 weeks. Tumor growth coincides with IL6 becoming detectable and increasing in blood, and tumors exhibit high expression of IL-4, IL-6, and IL-10 and are infiltrated by TAMs (CD11b+, F4/80+) and B lymphocytes (B220+, CD19+/IgM+). Immunofluorescence analyses demonstrate immunoglobulin deposition within the tumors. These data identify the NBL-Tag mice as the only known transgenic murine model for aggressive human mNBL MNA. Importantly, the pro-inflammatory microenvironment of the NBL-Tag tumors mimics that observed in human neuroblastoma. Our specific aims will allow us to identify the significance of a pro-inflammatory tumor microenvironment on neuroblastoma pathogenesis.

Body:

In last year's report we demonstrated our results for Task 1 and 2 of our SOW where we sought to understand the effect of B cells on tumor development. Our work demonstrated a modest effect of B cell ablation on tumor growth but did not alter the overall survival of NBL-Tag mice. This work was conducted using antibodies directed against the mouse B-cells (anti-mouse-CD20 antibody) with and without chemotherapy. In Task 3 we sought to determine the effects of macrophage elimination and the role of macrophages in tumor progression. We have also made significant findings related to Task 4 of the SOW in terms of role of IL6 in this system.

Our initial hypothesis was that tumor-associated macrophages (TAM) promote tumor growth early in the development of the NBL-Tag mice and are recruited to the tumor site after chemotherapy where they participate in promoting healing and growth processes. In series of timed experiments in the development of the NBL-Tag tumors, we assessed the presence of tumor associated macrophages in NBL-Tag mice and observed increase infiltration of macrophages in tumor tissue around 12 weeks of age which correspond to increase in tumor size by MRI imaging (Figure 1). We have also demonstrated that chemotherapy does not eliminate all the TAMs from the tumor site and the percentages of TAMs within the tumor microenvironment returns to baseline 14 days after chemotherapy (Figure 2).

We next attempted to remove the macrophages from the tumor microenvironment through treatment with liposomes packed with clodronate, which is known to induce macrophage apoptosis. The treatment was initiated after completion of a 5 day course of chemotherapy which mimics the regimen used in children with high risk neuroblastoma. However, there was little difference in the rate of tumor regrowth with or without clodronate compared to liposomes containing only phosphate buffer solution (Figure 2; $p = 0.1$). We also did not observe any difference in tumor growth in NBL-Tag mice treated with GW-2580, a CSFR1 inhibitor starting in

8 week old mice which is well before evidence of increase in tumor size by MRI (data not shown). We believe that our efforts to eradicate macrophages completely from the mice may not prove to be a feasible strategy and that tumor cells likely produce factors that protect macrophages and produce cytokines to redirect them after an injury (e.g. with chemotherapy).

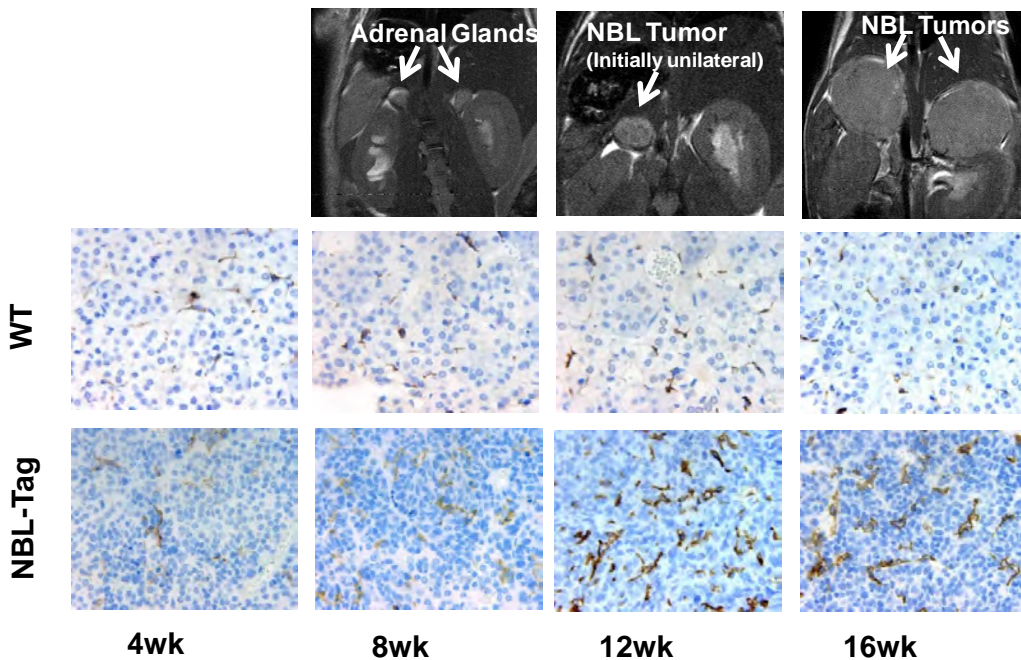


Figure 1. Infiltration of macrophages to the NBL-Tag tumors and expression of IL-6. NBL-Tag tumors stain positive for the F4/80 macrophage marker. Representative images of NBL-Tag tumors at different ages were stained with F4/80 specific antibodies by immunohistochemistry. Wt adrenal tissue was also stained to indicate basal levels of macrophage presence. F4/80 positive cells are indicated by brown color.

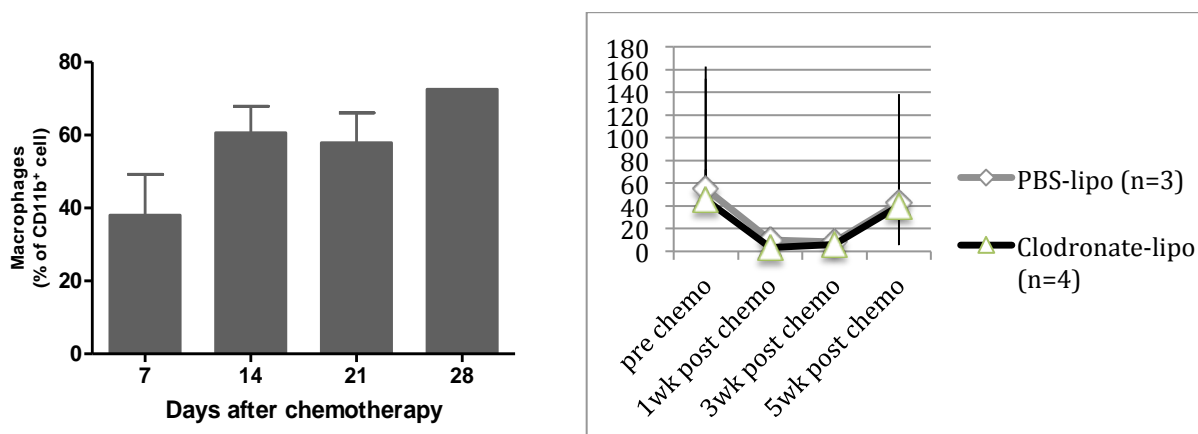


Figure 2. A) Macrophages are present within the tumors 7 days after chemotherapy and increase their infiltration by 14 days post chemotherapy. B) Clodronate loaded liposomes were injected immediately after completion of a 5 day course of chemotherapy with cyclophosphamide and topotecan. There was little difference in the growth of tumors in the clodronate arm compared to controls.

In an effort to study the mechanisms that promote tumor-TAM interactions and their effects, we have established *in vitro* and *in vivo* models that could be manipulated with pharmaceutical agents (Figure 3). The *in vitro* system utilizes the co-culture of peritoneal macrophages from wildtype C57B6 mice obtained through a magnetic bead separation and co-cultured with a NB-Tag cell line (NBT2). An increase in BRDU incorporation, which assess the rate of tumor cell proliferation, is observed in the co-culture system where tumor cell proliferation is nearly doubled with direct contact to macrophages or via a transwell system. The increase in proliferation is directly related to myelo-monocytic cells of the peritoneum (CD11b⁺ or F4/80⁺ cells) and only partially blocked with anti-IL6 antibody. This data suggests that the mechanism of action is only partially dependent on IL6 activity. *In vivo* studies confirmed the tumor-promoting effects of TAMs. Macrophages co-cultured with tumor cells enhanced growth of tumor cells in subcutaneous model, while macrophages obtained from peritoneum of tumor-bearing (NBL-Tag) mice which have been exposed to the tumor milieu, also enhanced tumor growth (Figures 3 C,D).

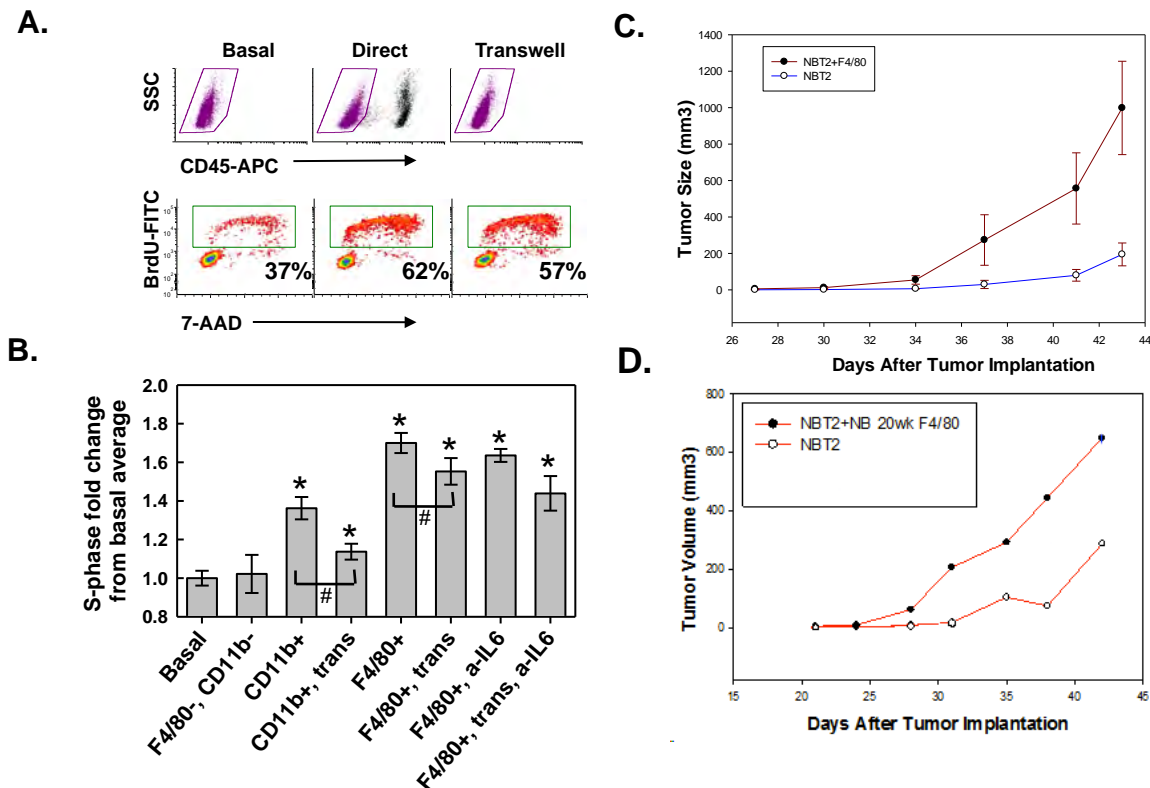


Figure 3. Macrophages enhance viability and proliferation of murine and human neuroblastoma cell lines independent of IL6 activity. A) Representative flow cytometry gating and BrdU assessment showing enhanced proliferation of NBT2 cell lines co-cultured directly (Direct) or using transwell system with peritoneal derived macrophages. The top panel illustrates the gating scheme used to exclude CD45 positive cells and the bottom panel shows contour plots of BrdU stained cells. Percentages indicate the population of cells in S-phase for each treatment. B) Average S-phase fold change of NBT2 cell lines compared to basal levels with or without anti IL6 neutralizing antibody (a-IL6) with direct co-culture, or transwell co-culture using non-monocytic derived cells (F4/80⁺CD11b⁺) or monocytic derived cells (CD11b⁺, F4/80⁺). The data were compiled from at least 3 independent experiments in triplicates; * indicate statistically significant p<0.05 difference from basal (NBT2 cell lines alone) and # indicates

statistical significance between indicated groups ($p < 0.05$). Asterisks (*) indicate a statistical significant ($p < 0.05$) difference between proliferation of neuroblastoma cells under basal condition versus during co-culture with macrophage. Abbreviations: peri: peritoneal, trans: transwell, a-IL6: anti-IL6 neutralizing antibody. C & D) Growth of tumor cells injected subcutaneously in immunodeficient mice (NSG) is enhanced by C) macrophages that have been trained via co-culture conditions with NBT2 cells, or D) by peritoneal macrophages obtained from 20 week old NBL-Tag mice with large tumor burdens (i.e. trained macrophages in vivo).

Our progress thus far has uncovered role of macrophages in the development and growth of NBL-Tag neuroblastoma tumors. Depletion studies have not inhibited this phenomenon, as it appears that total depletion of macrophages even for short periods of time is not successful. In ongoing experiments, we are crossing NBL-Tag mice with IL6 knockout mice to definitively understand the role of IL6, if any, in the growth of these tumors. We are also exploring strategies with pharmaceutical agents that reverse the polarization of macrophages rather than deplete them.

KEY RESEARCH ACCOMPLISHMENTS:

- TAMs are protected in the tumor microenvironment despite aggressive chemotherapy administration. The TAMs re-localize within 7-14 days into the tumor microenvironment.
- TAMs enhance tumor proliferation after being trained by tumor cells in 24 hours. The tumor-promoting effects are demonstrated in vivo and in vitro
- TAMs polarization that leads to acquiring tumor-promoting effects is only partially blocked by anti-IL6 therapy.

REPORTABLE OUTCOMES:

Abstracts:

Title: "Tumor-associated macrophages promote murine neuroblastoma tumor growth through upregulation of MYC and independent of IL6 expression." Michael Hadjidaniel, Sakunthala Muthgounder, Long Hung, Soheila Shirinbak, Michael Sheard, Hiroyuki Shimada, Shahab Asgharzadeh. American Association of Cancer Research, Pediatrics at the CrossRoads Meeting, 2013.

CONCLUSION

NBL-Tag is a robust mouse model of metastatic NBL-NA tumors recapitulating human disease. Our preliminary results reveal tumor-promoting effects of macrophages with NBL-Tag cell line. The tumor-promoting effect is only partially blocked by anti-IL6 therapy. We are continuing our work on Task 3 and 4 with crossing mice to IL6 knockout mice to demonstrate its role in tumor-promotion.

REFERENCES

None

APPENDICES

None